

**AN EXAMINATION OF THE EFFECTS OF SIMVASTATIN ON INNATE
IMMUNE RESPONSES TO *S. AUREUS***

A RESEARCH PAPER BY
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Materials and Methods

Mice

Male and female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed with unlimited access to standard laboratory chow and water until the start of each study. Mice were moved to individual filtered cages one day prior to study commencement and treatment administration. All treatment groups were subjected to a 12-hour day/night cycle and all experimental treatments were given at the same time of day for each treatment group. Maintenance of mice was performed by TES under the supervision of SAM and HAB. All murine experimental procedures have been approved and follow the regulations stated by the Animal Care and Use Committee (ACUC) at Ball State University.

Mice were between 8-10 weeks of age and ranged in body weight from 15-29 grams. All treatments were administered per body weight and dosages given accordingly. Mice were divided into subgroups, no less than three mice per treatment group, to create experimental conditions with variation in sex, age, and weight. The treatment groups were simvastatin (#567020-50MG, Calbiochem) pre-treated + *S. aureus*-infected (Simva +) and saline/ethanol pre-treated + *S. aureus*-infected (Simva -). All experimental groups were administered gentamicin antibiotic at 3, 6, 12, and 24 hours post *S. aureus* infection.

At the completion of each experimental set, mice were sacrificed using pentobarbital (10mg/ml) administered via intraperitoneal injection.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were maintained in 75cm² vented flasks containing filter sterilized M200 complete media (M-200-500, Cascade Biologicals) with Low Serum Growth Supplement (LSGS, S-003-10, Invitrogen) and incubated at 37°C with 5% CO₂. Media was replaced three times weekly and cells were sub-cultured upon reaching 60% confluency. Sub-cultured conditions were aseptically completed using 0.25% Trypsin-EDTA (#25200, Invitrogen). HUVEC were used for flow cytometric analysis of tissue factor (TF).

Staphylococcus aureus infection

S. aureus were sub-cultured from tryptic soy agar (TSA) slant stored at 4°C into tryptic soy broth (TSB) 3 days prior to murine infection. TSB was inoculated using sterile loop and placed overnight on shaker (225rpm) at room temperature (RT). Sub-cultures were performed, as previously described, each day until infection. Sub-cultures of *S. aureus* were performed at the same time each day (3-4pm) to ensure sufficient bacterial growth and experimental consistency.

On the day of infection, overnight sub-culture of *S. aureus* were mixed and separated into 4 equal aliquots. Aliquots were spun at 10,000 rpm, 37°C for 3 min., resuspended in pre-warmed sterile 0.85% saline, and re-spun as previously described. One aliquot was diluted in sterile saline and bacterial counts were made at 40X microscope objective using a hemacytometer. *S. aureus* was

diluted in 5% mucin to a bacterial count of 1×10^7 cells/ml for *in vivo* experimentation. All C57BL/6 mice were infected at time point 0 via intraperitoneal (i.p.) injection. *S. aureus* was diluted in sterile saline to a bacterial concentration of 3×10^8 cells/ml for *in vitro* experimentation.

In vivo treatments

C57BL/6 mice were pre-treated at 20 hours and 3 hours prior to *S. aureus* infection via i.p. injection. Pre-treatments were administered at the same time point for each experimental group. Pre-treatment consisted of i.p. injection of simvastatin (1000ng/g body weight (BW)) rehydrated in 100% ethanol (EtOH) or saline/EtOH vehicle control.

Antibiotic treatment with gentamicin (10mg/kg BW) was administered at 3, 6, 12, and 24 hours post *S. aureus* infection via i.p. injection. All treatment groups received antibiotic treatments. Murine specimens being sacrificed at 24 hours did not receive the 24 hour gentamicin injection.

Treatment groups included the experimental group, mice given simvastatin pre-treatment with gentamicin following *S. aureus* infection (Simva +) and the control group, mice given EtOH/saline pre-treatment with gentamicin following *S. aureus* infection (Simva -). Experimental collection was performed at 24 and 48 hours post *S. aureus* infection for each treatment group and mice were sacrificed via lethal i.p. dose of pentobarbitol.

Whole blood and serum was collected via cardiac puncture and used for bacterial clearance study and TNF- α enzyme linked immunosorbent assay (ELISA).

Clearance Study

Whole blood samples were collected via cardiac puncture at 24 and 48 hours post *S. aureus* infection. All samples were plated immediately following collection to prevent clot formation. Whole blood samples were diluted in sterile saline at 1:10 for bacterial clearance study 1 to ensure the isolation of colony forming units (CFU). All future studies did not require the dilution of whole blood samples. For clearance study 1, whole blood and dilution samples were plated on pre-warmed TSA/7.5% sodium chloride (NaCl) plates for selective growth of *S. aureus*. All future bacterial clearance studies utilized only whole blood samples. Plates were incubated overnight prior to the start of the clearance study to ensure that plates were not contaminated. Experimental plates were incubated overnight at 37°C with 5% CO₂. *S. aureus* bacterial colony counts were made following overnight incubation for each treatment and recorded for statistical analysis using SigmaStat and SigmaPlot.

TNF- α ELISA

TNF- α Enzyme-linked immunosorbent assay (ELISA, #88-7324, eBioscience) were used to detect TNF- α protein concentrations from C57BL/6 serum. Whole blood was isolated via cardiac puncture at 24 hour and 48 hours post *S. aureus*-infection, as previously described. Serum was separated at 4°C via centrifugation. Samples were run in a 96-well plate using manufacturer protein standards. Plate was coated with 1:250 dilution of capture antibody 24 hours prior to sample running, wells were washed with 1X PBS/ 0.05% Tween-20 (#P5927-100ML, Sigma) wash buffer, and incubated in 5X assay diluent. All well

rinses used same wash buffer. Two-fold serial dilutions of standard were performed to included concentration values of 0.5pg/ml - 1000pg/ml. Serum sample dilution were performed 1:5 to included values between 1:5-1:625. A dilution of Biotin-conjugate anti-mouse TNFa polyclonal detection antibody (1:250) and Avidin-HRP enzyme (1:250) were added to all samples. 10 M sulfuric acid (H₂SO₄) (2N) (#339741-100ML, Sigma) stop solution was used to halt reaction and plate was immediately read at 450nm using a Bio-Rad microplate reader. Treatment groups will be compared and data will be analyzed using One-way ANOVA with post-hoc analysis.

Flow cytometric analysis of TF in HUVEC

HUVEC were cultured in M200 as previously described and plated in 35-mm plates (Fisher Scientific #08-772 A) pre-coated with attachment factor (AF, S-006-100, Invitrogen) at 5×10^4 cells/plate and incubated at 37°C with 5% CO₂. Treatments groups consisted of cells pre-treated with simvastatin or dimethyl sulfoxide (DMSO, control, #D128-500, Fisher). Experimental groups were *S. aureus*-uninfected/ 0.01% DMSO, *S. aureus*-infected/ 0.01% DMSO, *S. aureus*-uninfected/ 1μM simvastatin, and *S. aureus*-infected/ 1μM simvastatin. HUVEC were pre-treated 24 hours post-initial plating procedure and incubated at 37°C with 5% CO₂.

HUVEC were infected with 3×10^8 -cfu *S. aureus* on day three following initial plating. Plates containing pre-treatment were washed in 1X sterile phosphate buffered saline (PBS, #20012-043, Invitrogen), replaced with 10% FBS/PBS for opsonization, and infected with *S. aureus* or saline control for 1

hour and 6 hours at 37°C with 5% CO₂. Following 1 hour infection, designated HUVEC were washed in 1X PBS and given 50 µg/ml of gentamicin (Sigma G 1272)/20 µg/ml lysostaphin (Sigma L-7386) in 10% FBS/PBS and returned to incubation.

All HUVEC treatments were harvested on ice at 6 hours post-infection. Cells were scraped, placed into pre-chilled FACS tubes, and spun at 1500rpm/4°C for 5 minutes. Anti-TF antibody CD142 (#550312, BD) was added to samples (excluding controls), samples were incubated on ice for 30 minutes. Following incubation, FACS buffer was added to cells, samples were spun as previously described, set with FACS and Fix, and analyzed via flow cytometric analysis.

Results

Previous studies indicated the ability of simvastatin to increase survival rates in *S. aureus* infected C57BL/6 mice. In order to establish a possible mechanism to explain this increased survivability, we wanted to look at possible innate immune responses to *S. aureus* in the presence of simvastatin. Bacterial clearance studies were performed as the first means to determine whether simvastatin increases survivability by enabling mice to clear *S. aureus* bacterial infection more efficiently than EtOH pre-treated control mice.

C57BL/6 mice, randomly assigned to treatment groups, consisted of male and female mice, 8-10 weeks of age. Whole blood isolated via cardiac puncture was plated on TSA / 7.5% NaCl agar plate for selective growth of *S. aureus* at 24 and 48 hours post infection. Colony forming units (cfu) were counted following 24 hour incubation at 37°C with 5% CO₂. Statistical analysis using SigmaStat/SigmaPlot found there to be no statistical significance in the number of *S. aureus* cfu in mice pre-treated with simvastatin or EtOH control (Figure 1). Results indicate that bacterial clearance is not enhanced in the presence of simvastatin at 24 or 48 hours post *S. aureus* infection in C57BL/6 mice. At 48 hours post infection, all *S. aureus* cleared regardless of the indicated treatment (Figure 1B).

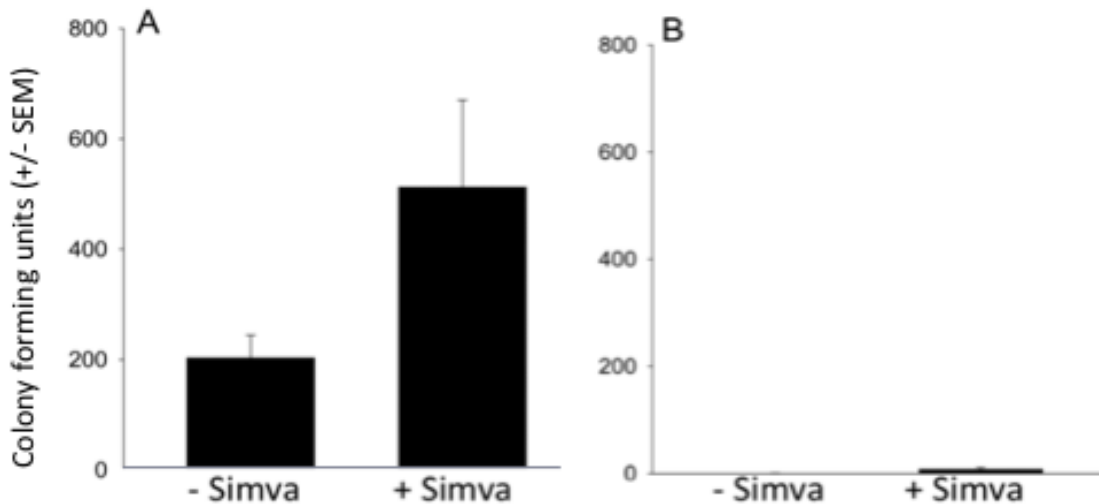


Figure 1. *Simvastatin* pre-treatment did not enhance *S. aureus* bacterial clearance in C57BL/6 mice. Mice (n=8-16 per group) were pre-treated with 1000ng/g simvastatin (+Simva) or given ethanol control (-Simva). Both treatment groups were given gentamicin (10mg/kg) post *S. aureus* infection. Whole blood was isolated from control and simvastatin-treated mice 24 hours (A) and 48 hours (B) post infection and plated on TSA plates. Colony forming units were counted on each plate after 24 hours. No statistical difference was determined in the number of colony forming units between treatment groups as determined by student's t-test.

Since clearance of *S. aureus* was not enhanced due to simvastatin pre-treatment, we evaluated innate immune responses to bacterial infection. TNF- α , a major pro-inflammatory cytokine involved in inflammation, was analyzed using ELISA techniques. Mice were experimentally divided as previously described. Serum was separated from whole blood extracted via cardiac puncture and used for TNF- α ELISA. At 24 hours (Figure 2A) and 48 hours (Figure 2B) post *S. aureus* infection, there is no

statistical difference in TNF- α concentration in mice pre-treated with simvastatin compared to mice pre-treated with EtOH control as evaluated by student's t-test. There is; however, an apparent immunological trend in the idea that simvastatin may play a role in decreasing TNF- α secretion, thereby reducing the inflammatory effects of infection. Simvastatin may promote the down regulation of innate immune responses, leading to decreased in pro-inflammatory cytokine secretion which decreases inflammation caused by *S. aureus* infection.

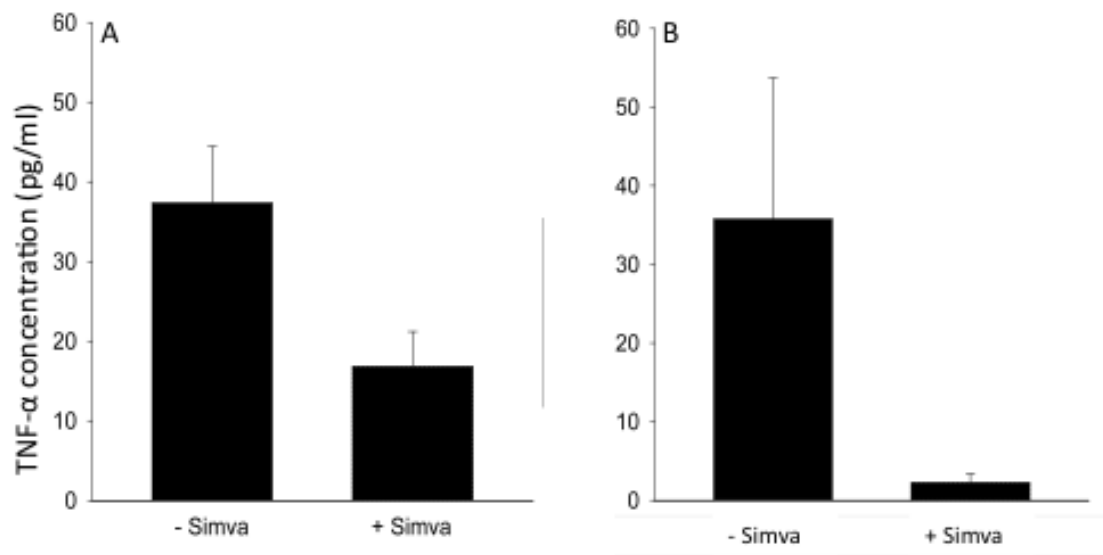


Figure 2. *Simvastatin pre-treatment does not significantly reduce TNF- α levels in *S. aureus*-infected C57BL/6 mice.* Mice were pre-treated with 1000ng/g simvastatin (+Simva) or given ethanol control (- Simva). Both treatment groups were given gentamicin (10mg/kg) post *S. aureus* infection. Serum was isolated all treatment mice at 24 hours (A) and 48 hours (B) post infection and used for TNF- α ELISA. No statistical difference was found in TNF- α concentration between treatment groups.

In vivo experimentation can be difficult due to variability. The last experiment in our current study involved *in vitro* analysis of TF in HUVEC. HUVEC pre-treated with simvastatin or DMSO control were evaluated for the presence of TF expression post *S. aureus* infection via flow cytometry. HUVEC were tagged with anti-TF antibody to determine if TF was present in more abundance on cells pre-treated with simvastatin as compared to the DMSO control. Flow cytometric analysis found that there is no decrease in TF expression in *S. aureus*-infected HUVEC pre-treated with simvastatin as compared to control (Figure 3, Figure 4). Flow cytometry determined there to be a significant increase (*) in TF expression in *S. aureus*-infected DMSO control cells as compared to uninfected cells (Figure 3). The trend indicates that *S. aureus* infection increases the expression of tissue factor. There was no difference found in TF expression between uninfected DMSO or uninfected simvastatin pre-treated HUVEC (Figure 3). The main result determined, indicates that simvastatin does not decrease the expression of TF in *S. aureus* infected HUVEC (Figure 4). Since TF induces the coagulation cascade, a reduction of TF expression in response to simvastatin might decrease the deleterious effects of over-induction of coagulation in response to *S. aureus* infection.

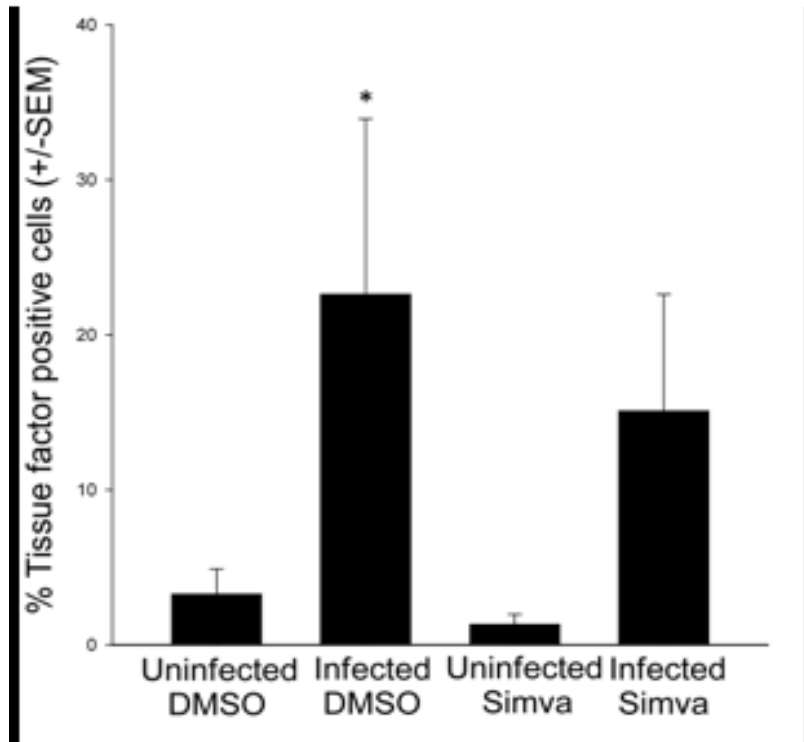


Figure 3. *Simvastatin pre-treatment does not decrease tissue factor expression in S. aureus-infected human HUVEC.* **S. aureus* infection increases the expression of tissue factor in DMSO cells. Human umbilical vein endothelial cells (HUVEC) were pre-treated with 1 μ M simvastatin (Simva) or 0.01% dimethyl sulfoxide (DMSO) for 24 hours prior to *S. aureus* infection. The percentage of tissue factor positive cells in the total cell population was measured via flow cytometry. No statistical difference was observed between infected cells pre-treated with simvastatin or DMSO as determined by one-way ANOVA with post hoc analysis. * $p < 0.05$

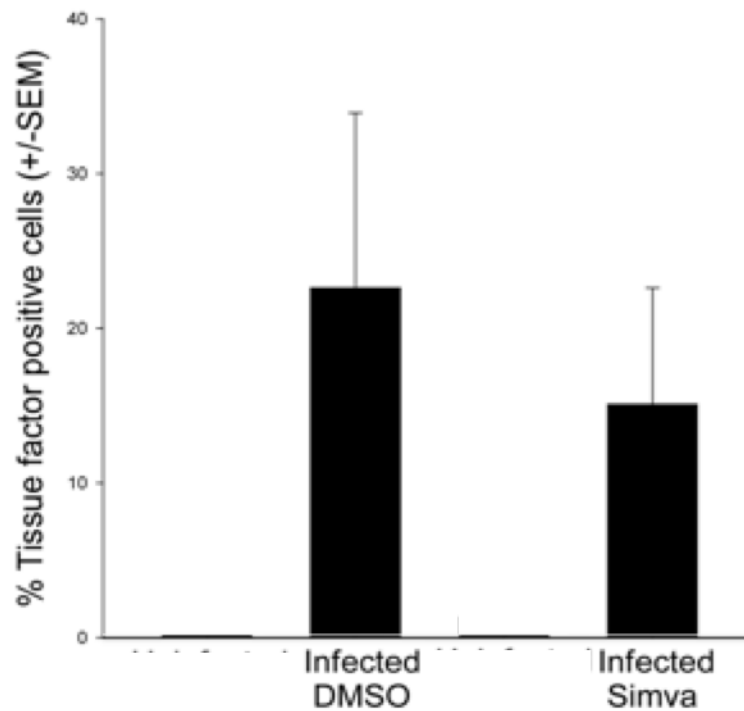


Figure 4. *Simvastatin pre-treatment does not significantly decrease tissue factor expression in S. aureus-infected human HUVEC cells.* Human umbilical vein endothelial cells (HUVEC) were pre-treated with 1 μ M simvastatin (Simva) or 0.01% dimethyl sulfoxide (DMSO) for 24 hours prior to *S. aureus* infection. The percentage of tissue factor positive cells in the total cell population was measured via flow cytometry. No statistical difference was observed between infected cells pre-treated with simvastatin or DMSO as determined by one-way ANOVA with post hoc analysis. Adapted from figure 3.

The results of our current study were not significant; however, simvastatin may be working to reduce the secretion of some major pro-inflammatory cytokines and mediators involved in the innate immune response to *S. aureus*. The cytokines and protein mediators affected by simvastatin may include TNF- α and TF. These mediators may have an effect on the resulting inflammatory responses produced from infection

with *S. aureus*. A closer look into the immunomodulatory role that statins play in innate immune responses to *S. aureus* may lead to a more conclusive understanding of the protective effects that simvastatin may have against sepsis.